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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 USC 371

International Application No.: PCT/BE99/00087
International Filing Date: July 6, 1999
Priority Date Claimed: July 6, 1998
Title of Invention: HIGH-THROUGHPUT SCREENING DIAGNOSTIC AND/OR DOSAGE METHOD OF AN AGONIST AND/OR AN ANTAGONIST FOR A CALCIUM-COUPLED RECEPTOR
Applicant(s) for DO/EO/US: Vincent Dupriez, Michel Detheux, Marc Parmentier

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. (X) This is a **FIRST** submission of items concerning a filing under 35 USC 371.

2. (X) This express request to begin national examination procedures (35 USC 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 USC 371(b) and PCT Articles 22 and 39(1).

3. () A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

4. (X) A copy of the International Application as filed (35 USC 371(c)(2))

   a) () is transmitted herewith (required only if not transmitted by the International Bureau).
   b) (X) has been transmitted by the International Bureau.
   c) () is not required, as the application was filed in the United States Receiving Office (RO/US).

5. (X) Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3))

   a) () are transmitted herewith (required only if not transmitted by the International Bureau).
   b) () have been transmitted by the International Bureau.
   c) () have not been made; however, the time limit for making such amendments has NOT expired.
   d) (X) have not been made and will not be made.

6. (X) A FIRST preliminary amendment.

7. (X) Other Items or information: 1) Six pages of drawings; 2) One page Abstract.

8. (X) A return prepaid postcard.

9. (X) The following fees are submitted:

				FEES
	BASIC FEE			$1,000
CLAIMS	**NUMBER FILED**	**NUMBER EXTRA**	**RATE**	
Total Claims	16 - 20 =	0 ×	$18	$0
Independent Claims	2 - 3 =	0 ×	$80	$0
	TOTAL OF ABOVE CALCULATIONS		$1000	
	TOTAL FEES ENCLOSED			$1000

10. (X) The fee for later submission of the signed oath or declaration set forth in 37 CFR 1.492(e) will be paid upon submission of the declaration.

11. (X) A check in the amount of $1000 to cover the above fees is enclosed.

12. (X) The Commissioner is hereby authorized to charge only those additional fees which may be required, now or in the future, to avoid abandonment of the application, or credit any overpayment to Deposit Account No. 11-1410. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

KNOBBE, MARTENS, OLSON & BEAR, LLP
620 Newport Center Drive
Sixteenth Floor
Newport Beach, CA 92660

Daniel Altman
Signature

Daniel E. Altman
Printed Name

34,115
Registration Number

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Dupriez, et al.	Group Art Unit Unknown
Int'l Appl. No.	:	PCT/BE99/00087	
Int'l Filing Date	:	July 6, 1999	
For	:	HIGH-THROUGHPUT SCREENING DIAGNOSTIC AND/OR DOSAGE METHOD OF AN AGONIST AND/OR AN ANTAGONIST FOR A CALCIUM-COUPLED RECEPTOR	
Examiner	:	Unknown	

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Preliminary to examination on the merits, please amend the above-captioned National Phase patent application as follows:

IN THE SPECIFICATION

On page 1, line 12, please insert --This is the U.S. National Phase under 35 U.S.C. §371 of International Patent Application PCT/BE99/00087, filed July 6, 1999.--.

On page 16, line 1, please cancel the word "CLAIMS" and substitute in its place -- WHAT IS CLAIMED IS:--.

IN THE CLAIMS

Please amend the claims as follows:

1. **(Amended)** **[Diagnostic and/or dosage]**A method for the identification of an agonist, **[and/or]** antagonist or any modulator **[for]**of a calcium-coupled **[receptor or a calcium-coupled channel or any other calcium-coupled]** protein, comprising **[the following successive steps]**:

-**[disposing the agonist and/or the antagonist upon]**immobilizing said modulator on a solid support,

-incubating one or more cell(s) expressing apoaequorin **[or any other related protein]** and said calcium-coupled **[receptor]**protein with **[coelenterazine or any other]**a cofactor of **[a calcium-sensitive]**said calcium-coupled protein in order to reconstitute an active aequorin **[by said cell(s)]**,

-adding **[to said solid support]** one or more of said cells, and

-**[obtaining the measurement of an emitted]**measuring the light emitted by said cell(s).

2. **(Amended)** **[Method]**The method according to claim 1, wherein the solid support is a microtiter plate.

3. **(Amended)** **[Method]**The method according to claim 2, **[characterised in that the]**wherein said microtiter plate is selected from the group consisting of: a 96-well microtiter plate, **[or]** a 384-well plate, **[or]**and a 1536-well plate **[1536-well-plate or any other format]**.

4. **(Amended)** **[Method]**The method according to **[any one of the preceding claims, characterized in that]**Claim 1, wherein the cell expresses apoaequorin in the cytoplasm or in the mitochondria **[or in any other part of the cell]**.

5. **(Amended)** **[Method]**The method according to **[any one of the preceding claims]**Claim 1, wherein the **[cell expressing a]** calcium-coupled **[receptor]**protein is **[a cell expressing an endogenous or]**a recombinant G-protein-coupled receptor **[and/or a cell which expresses proteins intended to ensure a coupling of the analysed receptor (endogenous or overexpressed) to the calcium pathway]**.

6. **(Amended)** **[Method]**The method according to claim 5, wherein said protein is selected from the group consisting of: **[natural]** Gα16 protein, **[or]** Gα15 protein, and a chimeric G-protein **[resulting from a fusion between two different G-proteins or phospholipase Cβ2 protein or any other coupling protein or chemical]**.

7. **(Amended)** **[Method]**The method according to **[any one of the preceding claims, characterised in that]**Claim 1, wherein the measurement of the emitted light is obtained

with one or more luminometer(s)**[, advantageously]** equipped with several dispensers and measurement heads.

8. **(Amended)** **[High throughput]**A high-throughput screening **[diagnostic and/or dosage]** device **[intended for the high-throughput screening diagnostic and/or dosage method according to any one of the preceding claim]**, comprising **[the following elements]**:

-a microtiter plate**[, preferably a 96-well microtiter plate]**,

-a medium containing cell(s) expressing apoaequorin and a calcium-coupled **[receptor]**protein,

-**[a medium containing]** coelenterazine, and

-**[means for detecting an]**a detector of emitted light by said cell(s).

9. **(Amended)** **[Device]**A device according to claim 8, further comprising **[means for the automatic performance of the successive steps of the diagnostic and/or dosage method according to any one of the claims 1 to 7]**an automated mechanism which can perform the method.

10. **(Amended)** **[Agonist]**An agonist or antagonist of a receptor identified by the method according to **[any one of the claims 1 to 8]**Claim 1.

Please add the following Claims:

11. The method of Claim 1 wherein the calcium-coupled protein is a calcium-coupled receptor.

12. The method of Claim 1 wherein the cofactor of the calcium-coupled protein is coelenterazine.

13. The method according to Claim 1, wherein the cell expresses proteins intended to ensure a coupling of the analysed receptor to the calcium pathway.

14. The method according to claim 5, wherein said chimeric G-protein results from a fusion between two different G-proteins.

15. The method according to claim 5, wherein said chimeric G-protein results from a fusion between a G-protein and phospholipase Cβ2.

16. The method of Claim 8 wherein said calcium-coupled protein is a calcium-coupled receptor.

Int'l Appl. No. : PCT/BE99/00087
Int'l filing Date : July 6, 1999

IN THE ABSTRACT

Please add the enclosed abstract at page 18 to the Application.

REMARKS

The claims and specification have been amended to correspond to the rules of practice before the United States Patent and Trademark Office. Support for added claims 11-16 can be found in the claims as filed.

Conclusion:

Should there be any questions, the Examiner is respectfully requested to contact the undersigned attorney at the telephone number below.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 8 Jan. 2007

By: Daniel Altman

Daniel E. Altman
Registration No. 34,115
Attorney of Record
620 Newport Center Drive
Sixteenth Floor
Newport Beach, CA 92660
(949) 760-0404

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HIGH-THROUGHPUT SCREENING DIAGNOSTIC AND/OR DOSAGE METHOD OF AN AGONIST AND/OR AN ANTAGONIST FOR A CALCIUM-COUPLED RECEPTOR

Field of the invention

The present invention is related to a high-throughput screening diagnostic and/or dosage method and device of an agonist and/or an antagonist for a calcium-coupled receptor and the agonist and/or antagonist of said calcium-coupled receptor identified by said method and device.

Background of the invention and state of the art

A lot of G-protein-coupled receptors (GPCR) trigger, upon binding of an agonist, a transient increase in intracellular calcium concentration. This variation acts as an internal secondary messenger and is an important modulator of many physiological mechanisms (reviewed by Rink (1990), Tsunoda (1993) and by Santella & Carafoli (1997)). Measurement of intracellular calcium concentration in cells expressing a GPCR can thus be used to monitor the efficacy of activation of a GPCR by various compounds known - or suspected - to be a ligand for this GPCR.

The activation of other receptors such as ions channels may also induce an intracellular calcium concentration.

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Changes in calcium concentration can be detected by several means and methods, like the use of fluorescent dyes (for example: fura-2, fluo-3, fluo-4 and indo-1).

However, Ca^{++} sensitive dyes have limitations. Activation of the dyes with an excitation beam requires complicated and expensive instruments and limits the use of the plastic labware such as microtiter plates.

Another method for intracellular calcium concentration measurement is the use of cell lines overexpressing a GPCR and apoaequorin, such as described by Sheu et al. (1993). In this system, cells expressing apoaequorin are incubated with coelenterazine, which is the co-factor of aequorin. During this incubation, coelenterazine enters the cell and conjugates with apoaequorin to form aequorin, which is the active form of the enzyme. Upon incubation of the cells with an agonist of the GPCR, intracellular calcium concentration increases. This increase leads to the activation of the catalytic activity of aequorin, which oxidises coelenterazine and yields apoaequorin, coelenteramide, CO_2 and light. Once the photon has been emitted, the complex must dissociate and apoaequorin must recombine with a new coelenterazine molecule to be able to emit light again. Thus, in this system, measurement of light emission following agonist addition reflects its ability to activate the GPCR and thus to increase intracellular calcium concentration. Because light is emitted only during 20 to 30 seconds after activation of the GPCR, recording of the emitted light must be performed during the few seconds following agonist addition to the cells. This flash-type signal is due to the fact that (1) intracellular calcium increase triggered by GPCR is only transient and (2) as mentioned earlier, after

oxidation of coelenterazine, apoaequorin must recombine with coelenterazine to be able to emit light again.

The Patent Application EP-0341477 teaches the expression of jellyfish photoprotein aequorin in a mammalian cell system by cloning gene pAQ440 specifying the biosynthesis of the aequorin into an expression vector plasmid of a mammalian cell system, subjecting the resulting plasmid to transfection and producing the photoprotein aequorin in the mammalian cell.

The Patent US-5,422,266 describes a gene encoding apoaequorin protein included in a vector capable of expressing the apoaequorin in E. coli.

The Patent US-5,714,666 describes mammalian cell lines or transgenic animals expressing apoaequorin and a receptor involved in the modulation of intracellular calcium. This document also describes a method of measuring intracellular calcium comprising adding coelenterazine cofactors to said mammalian cells expressing apoaequorin and measuring photoemission where emission of photons is indicative of intracellular calcium concentration.

However, the methods of the state of the art require firstly the spreading of cells from a mammalian cell line expressing apoaequorin on a solid support (for example a 96-well plate), secondly the addition of the coelenterazine cofactor upon the cells and incubation to reconstitute a functional aequorin, thirdly the preparation of the agent affecting a receptor involved in the modulation of intracellular calcium concentration, and its addition to the prepared cells, and finally the measurement of the photoemission.

Furthermore, as mentioned above, light is emitted only during 20 to 30 seconds after activation of the GPCR. Therefore, the recording of the emitted light

must be performed during the few seconds following agonist addition to the cells.

Therefore, the methods used in the state of the art are not adequate for a detection based upon high-throughput screening level, which usually need luminometer(s) and require the use of microtiter plates for the testing of thousands of compounds.

Aims of the invention

The present invention aims to provide a method and means, which do not present the drawbacks of the state of the art, for detecting biologically active substances, especially agonists and/or antagonists for calcium-coupled receptors.

A main aim of the present invention is to provide such method and means which allow the detection of biological active substances at a high-throughput scale, which could be adapted to specific recipients such as microtiter plates without requiring the modification of the high-throughput screening device.

Another aim of the present invention is to provide an easy and non-expensive method that could be easily automated.

Summary of the invention

The present invention is related to a high-throughput screening diagnostic and/or dosage method of an agonist and/or an antagonist for a "calcium-coupled" receptor, comprising the following successive steps:

- disposing an agonist and/or an antagonist (preferably of a molecule) of said receptor upon a solid support,
- incubating one or more cell(s) expressing apoaequorin and said "calcium-coupled" receptor with coelenterazine

in order to reconstitute an active aequorin by said cell(s),

- adding to said solid support one or more of said cells, and
- obtaining the measurement of an emitted light by said cell(s).

The term "calcium-coupled" receptor means any receptor (such as a G-coupled receptor or an ion channel) whose activation (by an ion, a known or unknown agonist or antagonist molecule) may increase an intracellular calcium concentration in the cell comprising said receptor, preferably in its cytoplasmic membrane.

The terms "disposing... upon a solid support" means the step of putting said compound into a recipient such as a microtiter plate or any other solid support without requiring any (covalent or other) fixing of said compound to said solid support.

Advantageously, the solid support is a microtiter plate, preferably a 96-well microtiter plate.

Advantageously, the cell expressing apoaequorin and the calcium-coupled receptor is a cell expressing a G-coupled receptor and possibly one or more protein(s) intended to ensure a coupling of the receptor to the calcium pathway.

Preferably, said protein is selected from the group consisting of a natural Gα16 or Gα15 protein, a chimeric G-protein resulting from a fusion between two different G-proteins or a phospholipase Cβ2 protein.

The measurement of the emitted light is advantageously obtained with one or several luminometer(s), possibly equipped with several dispensers and measurement heads.

The present invention is also related to a high-throughput screening diagnostic and/or dosage device intended for the diagnostic and/or dosage method according to the invention, said device comprising the following elements:

- a recipient, preferably a microtiter plate, more preferably a 96-well microtiter plate,
- a medium containing cell(s) expressing apoaequorin and a calcium-coupled receptor,
- a medium containing coelenterazine, and
- means (such as one or more luminometer(s) equipped with one or several dispensers and measurement heads) for detecting and possibly quantifying an emitted light by said cell(s).

Advantageously, the device according to the invention comprises means for the automatic performance of the successive steps of the diagnostic and/or dosage method according to the invention.

A last aspect of the invention is related to the agonist and/or the antagonist of a calcium-coupled receptor identified by the method or the device according to the invention.

The present invention will be described in details in the following non-limiting examples, in reference to the enclosed figures.

Brief description of the drawings

Figure 1 shows a series curves representing the intensity of the emitted light by cells as a function of time for each well of a 96-well plate injected with cells expressing the CCR5 receptor, apoaequorin and Gα16. The scaling is the same for all the graphs. Recording of

the signal was performed for 30 seconds. Ligand concentrations are increasing from column 1 towards column 12. All measurements were performed in duplicate: lines A and B: ligand is RANTES; lines C and D: ligand is MIP-1α; lines E and F: ligand is MIP-1β; lines G and H: ligand is derivative A of RANTES.

Figure 2 represents the dose-response curve for different agonists of the CCR5 receptor which represent RLU (integration of emitted light on 30 seconds) according to the logarithm of the final concentration of the ligand.

Figure 3 represents the dose-response curve for different agonists of the 5HT-2B receptor.

Figure 4 represents the dose-response curve of the light emitted from K562 cells expressing CCR3 and aequorin as a response to the activation of the receptor by eotaxin.

Figure 5 represents the dose-response curves for cells expressing aequorin and Gα16 and (panel A) the orexin 1 receptor or (panel B) the orexin 2 receptor.

Figure 6 represents the dose-response curve for different antagonists of the 5HT-2B receptor.

Description of a preferred embodiment of the present invention

Detection of agonistic activities by means of mammalian cell lines expressing apoaequorin and a GPCR requires the measurement of the emitted light to be performed just after placing the cells in contact with the potential agonist. This can easily be measured at low

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throughput using a single-tube luminometer. However, up to now, this biological system could not be used at a high-throughput scale. Indeed:

(1) the necessity to measure light just after placing the cells in contact with the agonist to be tested compels to use a luminometer equipped with a build-in dispenser. For example, due to the short duration of light emission, it is impossible to inject the drugs to be tested on the cells placed in the 96 wells while the plate is outside the luminometer and to subsequently record emitted light with the plate into the luminometer. Even if the plate could rapidly (i.e. in less than 15 seconds) be placed into the luminometer after injection of the drugs to be tested, current apparatus do not allow the measurement of light from the 96 wells before the extinction of the flash signal of aequorin, as these luminometers are not equipped with 96 detectors.

(2) luminometers equipped with a build-in dispenser only allow to inject a single solution into the 96 wells, making it impossible to inject a different drug in each well. Moreover, the washing of the dispenser before each measurement, for the injection of another drug in the next well, is time-consuming and thus is not suitable for the high-throughput scale. The same problem occurs with devices equipped with 6 dispensers (e.g. the "Microbeta Jet" from EG&G Wallac) as the 6 dispensers only deliver a single solution.

The present invention provides a method for performing high-throughput screening of drugs binding to GPCR by the use of mammalian cell lines expressing apoaequorin and a GPCR and by the use of a conventional luminometer. Following this method, the solutions to be tested for (ant)agonistic activities are placed in the

wells of a 96-well plate. Cells expressing apoaequorin and a GPCR are detached from the culture plate (or collected from suspension cultures) and are incubated with coelenterazine to reconstitute active aequorin. These are then maintained in suspension with a magnetic stirrer and the cell suspension is injected, well by well, on the solutions of potential agonist to be tested. Light emission is then recorded for 1 (alternatively up to 30 or more) seconds. This method, by injecting the same cell suspension in each of the 96 wells, avoids the need of washing the dispenser between each measurement and allows to perform 96 measurements of agonist-induced aequorin light emission in 15 minutes or less with a single dispenser luminometer. Alternatively, it allows to perform 96 measurements of agonist-induced aequorin light emission in 2 minutes or less with a luminometer equipped with 6 dispensers and measurement heads (e.g. with the "Microbeta Jet" from EG&G Wallac).

This method thus allows to perform high-throughput screening (10 000 samples/day) with mammalian cell lines expressing apoaequorin and a GPCR and by the use of a conventional luminometer. This reduces the screening time and the amount of drugs needed for each measurement.

This system also allows to perform a functional screening with very few (down to 5000 or less) cells per measurement.

The injection of the cells into the wells containing the agonists did not increase the background of the measurement (that could for example have originated from cell breakage, releasing aequorin molecules from the cells into the culture medium, where the calcium concentration would have triggered the emission of light from aequorin). A signal-to-noise ratio above 50 was commonly obtained with this system of cell injection.

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The method according to the invention is suitable for performing high-throughput analysis of GPCR or other calcium-coupled-receptor stimulation by known or potential agonists by means of cells expressing the receptor and apoaequorin. These cells may express apoaequorin in the cytoplasm, as described by Sheu et al. (1993) or Button and Brownstein (1993) or may express apoaequorin in the mitochondria, by means of the addition of a mitochondrial targeting sequence to the aequorin, as used by Stables et al. (1997) or in any other part of the cell. These cells may also express proteins intended to ensure coupling of the over-expressed receptor to the calcium pathway. These may be the natural Gα16 or Gα15 proteins (Milligan et al., 1996), chimeric G proteins resulting from a fusion between two different G proteins (Komatsuzaki et al., 1997), phospholipase C-β2 (Park et al., 1992), or any other "universal coupling" protein.
Once the cells have been prepared and loaded with coelenterazine, they can be used for several hours (at least 9 hours). The load in coelenterazine and the intensity of the light emitted by the cells upon agonist stimulation lasts is stable for this period of time.

Examples

Example 1

A CHO cell line expressing the chemokine CCR-5 receptor, the Gα16 coupling protein and apoaequorin was established. Cells were cultivated as a monolayer in HAM'sF12 medium containing 10% Foetal bovine serum (FBS). On the day of the experiment, the culture medium was removed and cells were incubated for 5 min at room temperature in PBS-EDTA (phosphate buffered saline solution without calcium, supplemented with 5 mM EDTA). Cells were

detached from the culture vessel by shaking the culture plate by hand and by pipetting up and down. Cells were centrifuged and the supernatant was removed to eliminate the EDTA; the pellet was resuspended in HAM's F12 culture medium without FBS and with 0.1% Bovine Serum Albumin. Cells were counted by means of a Thomas cell, were centrifuged again and were resuspended in HAM's F12 culture medium without FBS and with 0.1% Bovine Serum Albumin at a concentration of 5.10^6 cells/ml. Coelenterazine (or a derivative of it , e.g. Coelenterazines f, h, n, cp or hcp, from Molecular Probes Inc.) at 500 µM in methanol was added to the cell suspension at a final concentration of 5 µM. The cell suspension was then stored in the dark at room temperature for 3 to 5 h, with shaking every 15 to 30 min to maintain the cells in suspension.

Series of dilutions of known ligands were prepared in HAM's F12 culture medium without FBS and with 0.1% Bovine Serum Albumin and 50 µl of each of these solutions were placed in the wells of a 96-well plate. The cell suspension was diluted 5 times with medium HAM'sF12 without FBS and with 0.1% Bovine Serum Albumin and was placed in a glass or plastic container protected from light by wrapping it with aluminium foil. A magnetic stirring bar was added to the suspension and a magnetic stirrer was used at low speed (1 to 5 rounds per second) to maintain the cells in an homogenous suspension. The magnetic stirring bar was equipped with a ring to avoid crushing the cells, and the subsequent release of aequorin in the culture medium. Alternatively, a culture vessel equipped for culture of cells in suspension may be used.

One uses the EG&G Wallac's MicroLumat-Plus microplate luminometer, which allows injection and direct subsequent recording of the emitted light from each well of

a 96-well plate. The end of the entrance tube of the dispenser was placed at the bottom of the cell suspension and the dispenser was washed with 3 times the dead volume of the apparatus so that all the volume of the tube and pumps was filled with the cell suspension. The 96-well plate containing the solutions of agonists was then inserted into the luminometer. Then, for each well, 50 µl of the cell suspension (i.e. 100 000 cells) was dispensed into the well (at the lowest injection speed (0.4 s) to prevent cell breakage that would release aequorin into the culture medium) and the emitted light was immediately recorded during 30 seconds. After reading the first well, cells were injected into the next well and emitted light was recorded, etc. For each plate, a series of curves representing the intensity of the emitted light as a function of time for each well was displayed (figure 1). The intensity of the emitted light was integrated over 30 s using the Winglow software provided with the luminometer, yielding for each well one value representative of the emitted light and hence of the stimulation of the CCR-5 receptor by the agonist present in the well. These values can be plotted against the logarithm of the ligand concentration to generate dose-response curve as shown in figure 2. These allow the determination of half-maximal response doses (EC_{50}) for each ligand. For the generation of these data, 288 measurements were performed in less than 3 hours.

Example 2

A CHO cell line expressing the serotonin 5HT-2B receptor, the Gα16 coupling protein and apoaequorin was established. Cells were treated as described in Example 1 and were dispensed after dilution (100 µl/well,

corresponding to 50 000 cells) on 100 µl of solutions of known agonists for this receptor. The emitted light was recorded during 20 s for each well. Dose-response curves obtained for different agonists are shown in figure 3. For the generation of these data, 144 measurements were performed in less than 1 hour.

Example 3

K562 cells expressing the chemokine CCR3 receptor were transfected by a plasmid for the expression of aequorin and the Gα16 coupling protein. Cells stably transfected were selected for 2 weeks with the antibiotic Zeocin. These cells were cultured in suspension in DMEM culture medium containing 10% FBS. They were centrifuged and the pellet was used as described in example 1 to perform aequorin measurements. A dose-response curve with eotaxin and MCP-4 generated by this method is described in figure 4.

Example 4

A CHO cell line expressing the orexin 1 or orexin 2 receptor, the Gα16 coupling protein and apoaequorin was established. Cells were treated as described in Example 1 and were dispensed after dilution (100 µl/well, corresponding to 25 000 cells) on 50 µl of solutions of known agonists for these receptor. The emitted light was recorded during 20 s for each well. A dose-response curve obtained with this method is showed in figure 5 A.

Example 5

A CHO cell line expressing the canabinoïd CB1 receptor, the Gα16 coupling protein and apoaequorin was

established. Cells were treated as described in Example 1 and were dispensed after dilution (100 µl/well, corresponding to 50 000 cells) on 100 µl of solutions of a known agonist for this receptor. The emitted light was recorded during 20 s for each well. A dose-response curve obtained with this method is showed in figure 5 B.

For the measure of antagonistic activities, cells were injected on series of dilutions of antagonists. At this stage, the emitted light was recorded to check that the potential antagonists had no agonistic activity. The cells were incubated with the antagonists for 30 min. A solution of agonist (alpha-methyl-5HT) of the 5HT-2B receptor was then injected on the cells and the emitted light was immediately recorded for each well. The emitted light was plotted as a function of the logarithm of the antagonist concentration to yield the graph shown. Increasing antagonist concentrations result in a decreasing light emission upon agonist addition. An agonist of another receptor expressed by the cells (usually ATP, acting at P2 receptors) can then be injected on the mixture of antagonist, cells and agonists as a control to check that the cells still have active aequorin up to that moment of the experiment. For example, cytotoxic compounds that increase the intracellular calcium concentration can make the aequorin consume all the coelenterazine present in the cell. Such a cytotoxic compound will be detected by the absence of signal upon ATP injection (Fig. 6).

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CLAIMS

1. Diagnostic and/or dosage method of an agonist and/or an antagonist or any modulator for a calcium-coupled receptor or a calcium-coupled channel or any other calcium-coupled protein, comprising the following successive steps:

- disposing the agonist and/or the antagonist upon a solid support,
- incubating one or more cell(s) expressing apoaequorin or any other related protein and said calcium-coupled receptor with coelenterazine or any other cofactor of a calcium-sensitive protein in order to reconstitute an active aequorin by said cell(s),
- adding to said solid support one or more of said cells, and
- obtaining the measurement of an emitted light by said cell(s).

2. Method according to claim 1, wherein the solid support is a microtiter plate.

3. Method according to claim 2, characterised in that the microtiter plate is a 96-well microtiter plate, or a 384-well plate, or a 1536-well-plate or any other format.

4. Method according to any one of the preceding claims, characterised in that the cell expresses apoaequorin in the cytoplasm or in the mitochondria or in any other part of the cell.

5. Method according to any one of the preceding claims, wherein the cell expressing a calcium-coupled receptor is a cell expressing an endogenous or recombinant G-protein-coupled receptor and/or a cell which expresses proteins intended to ensure a coupling of the

analysed receptor (endogenous or overexpressed) to the calcium pathway.

6. Method according to claim 5, wherein said protein is selected from the group consisting of natural Gα16 or Gα15 protein, chimeric G-protein resulting from a fusion between two different G-proteins or phospholipase Cβ2 protein or any other coupling protein or chemical.

7. Method according to any one of the preceding claims, characterised in that the measurement of the emitted light is obtained with one or more luminometer(s), advantageously equipped with several dispensers and measurement heads.

8. High-throughput screening diagnostic and/or dosage device intended for the high-throughput screening diagnostic and/or dosage method according to any one of the preceding claims, comprising the following elements:

- a microtiter plate, preferably a 96-well microtiter plate,
- a medium containing cell(s) expressing apoaequorin and a calcium-coupled receptor,
- a medium containing coelenterazine, and
- means for detecting an emitted light by said cell(s).

9. Device according to claim 8, comprising means for the automatic performance of the successive steps of the diagnostic and/or dosage method according to any one of the claims 1 to 7.

10. Agonist or antagonist of a receptor identified by the method according to any one of the claims 1 to 8.

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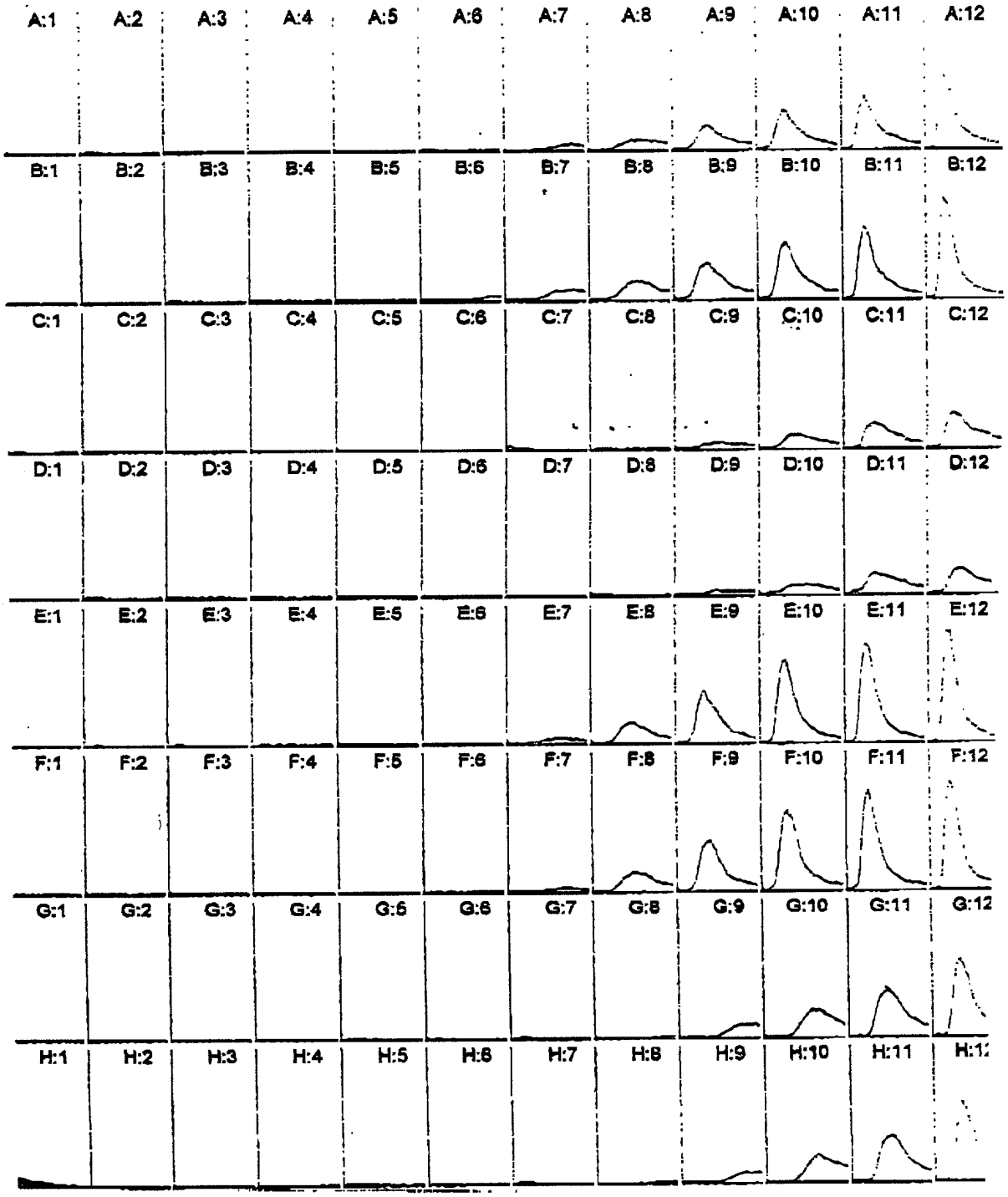
ABSTRACT

The present invention is related to a diagnostic an/or dosage method of an agonist and/or an antagonist and/or a modulator for a calcium-coupled receptor and/or channel and/or any other calcium-coupled protein, comprising the following successive steps: disposing the agonist and/or the antagonist and/or the modulator upon a solid support; Incubating one or more cell(s) expressing apoaequorin and said calcium-coupled receptor with coelenterazine in order to reconstitute an active aequorin in said cell(s). The present invention is also related to the diagnostic and/or dosage device intended for the method according to the invention.

FIG.1



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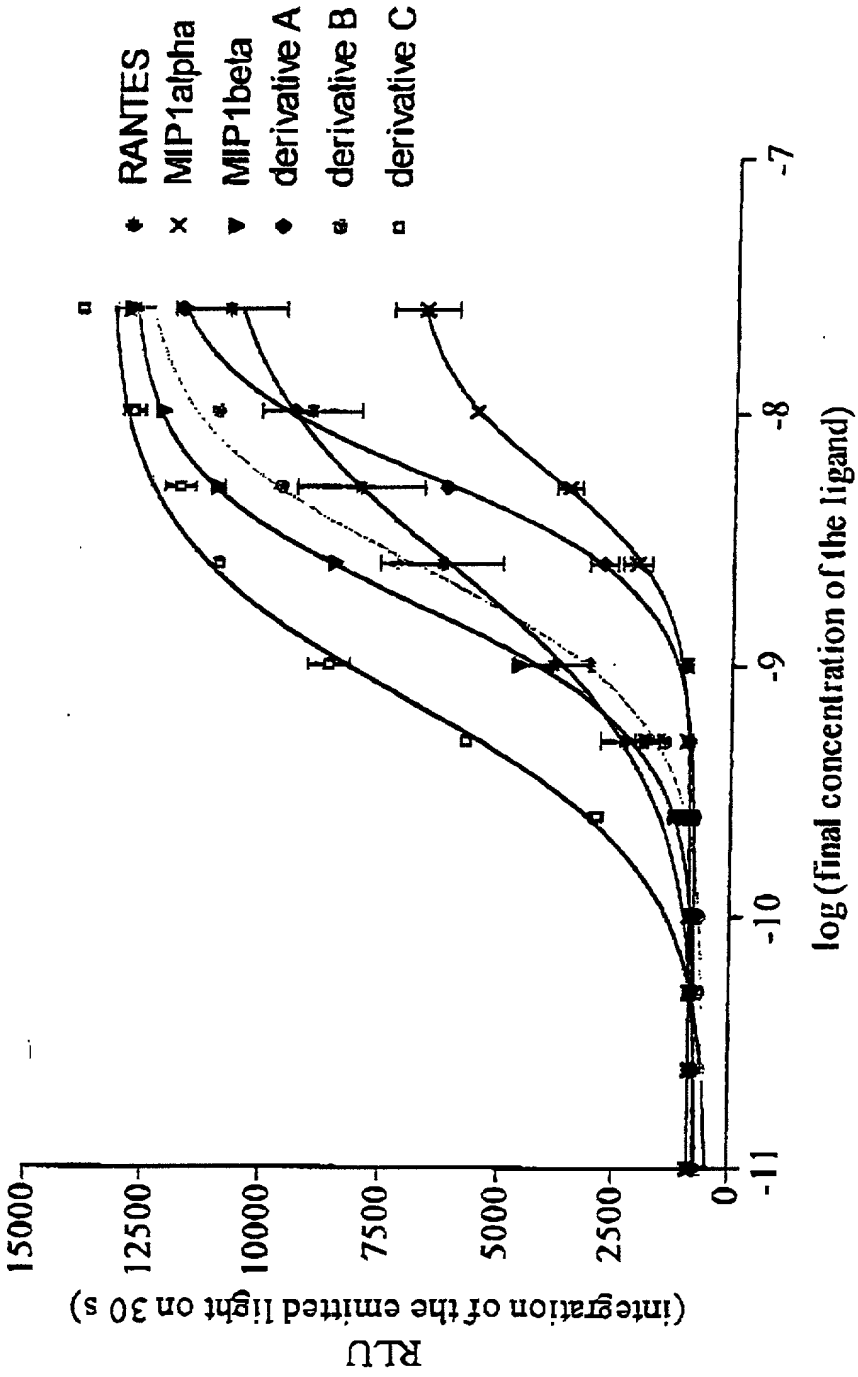
RLU
(integration of the emitted light on 30 s)
15000
12500
10000
7500
5000
2500
0
-11
-10
-9
-8
-7
log (final concentration of the ligand)
RANTES
MIP1alpha
MIP1beta
derivative A
derivative B
derivative C

FIG. 2

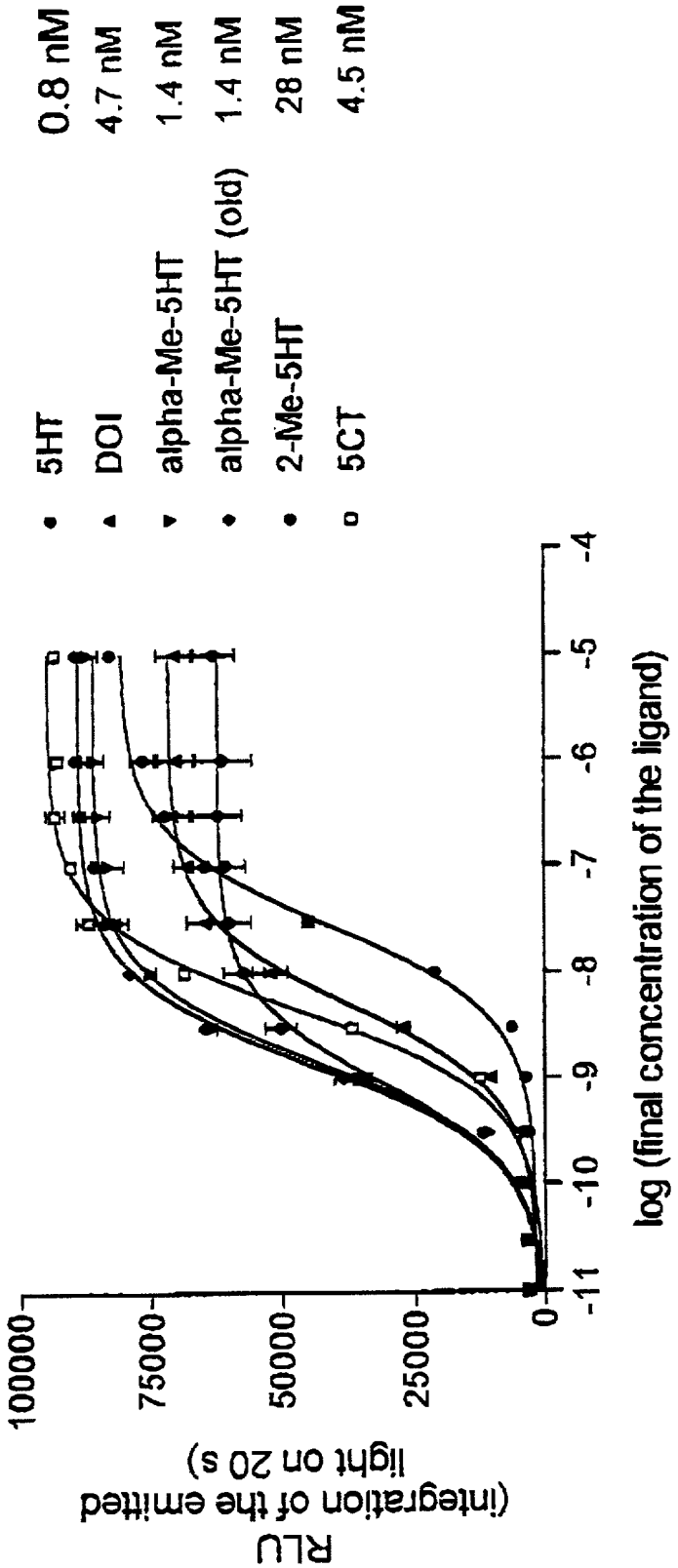
RLU
(integration of the emitted light on 20 s)
100000
75000
50000
25000
0
-11
-10
-9
-8
-7
-6
-5
-4
log (final concentration of the ligand)
5HT 0.8 nM
DOI 4.7 nM
alpha-Me-5HT 1.4 nM
alpha-Me-5HT (old) 1.4 nM
2-Me-5HT 28 nM
5CT 4.5 nM

FIG. 3

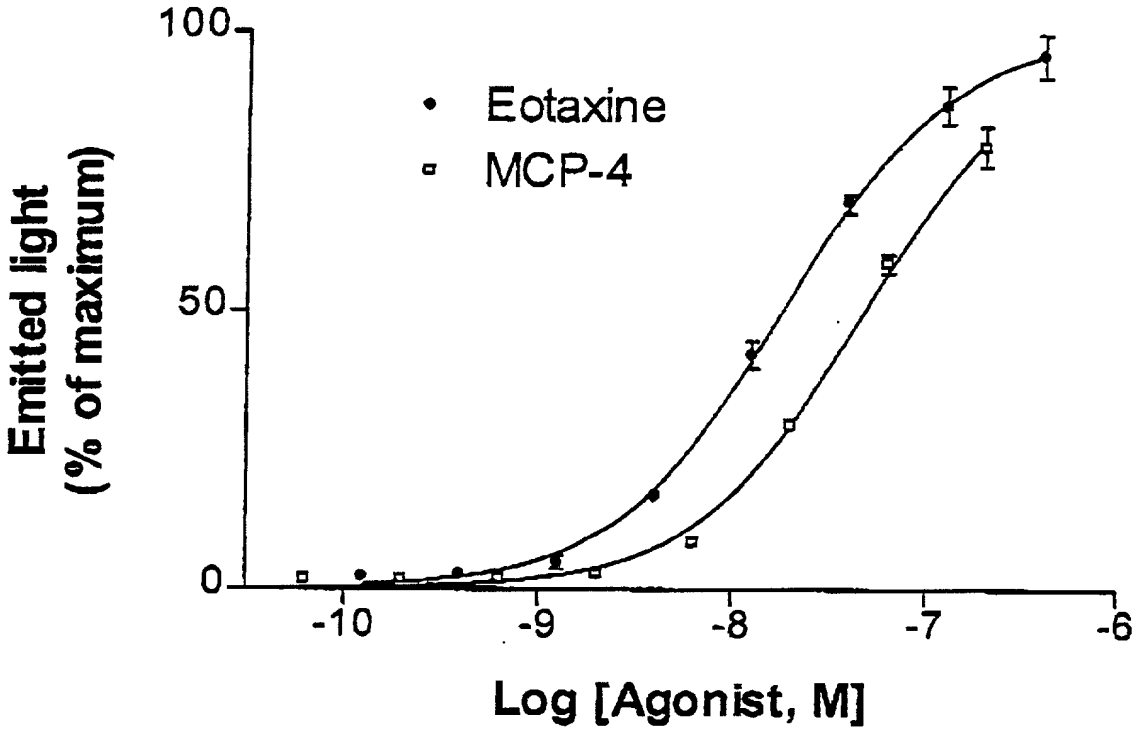

FIG. 4

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A.

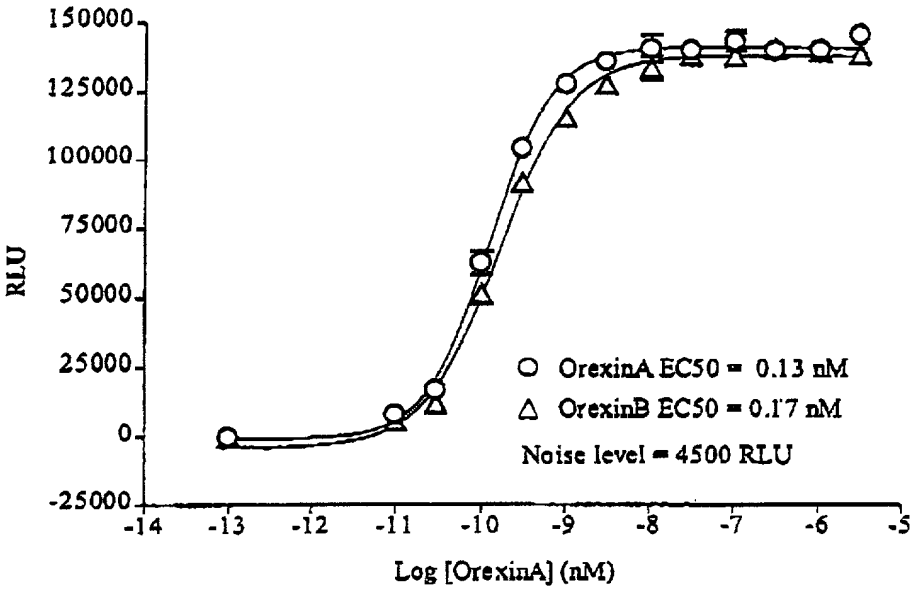

FIG. 5A

B.

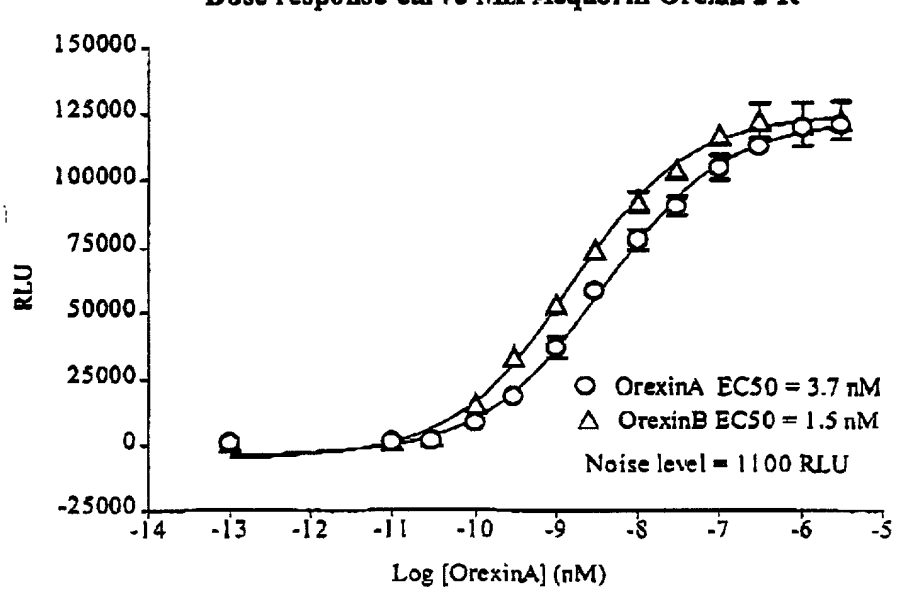

FIG. 5B

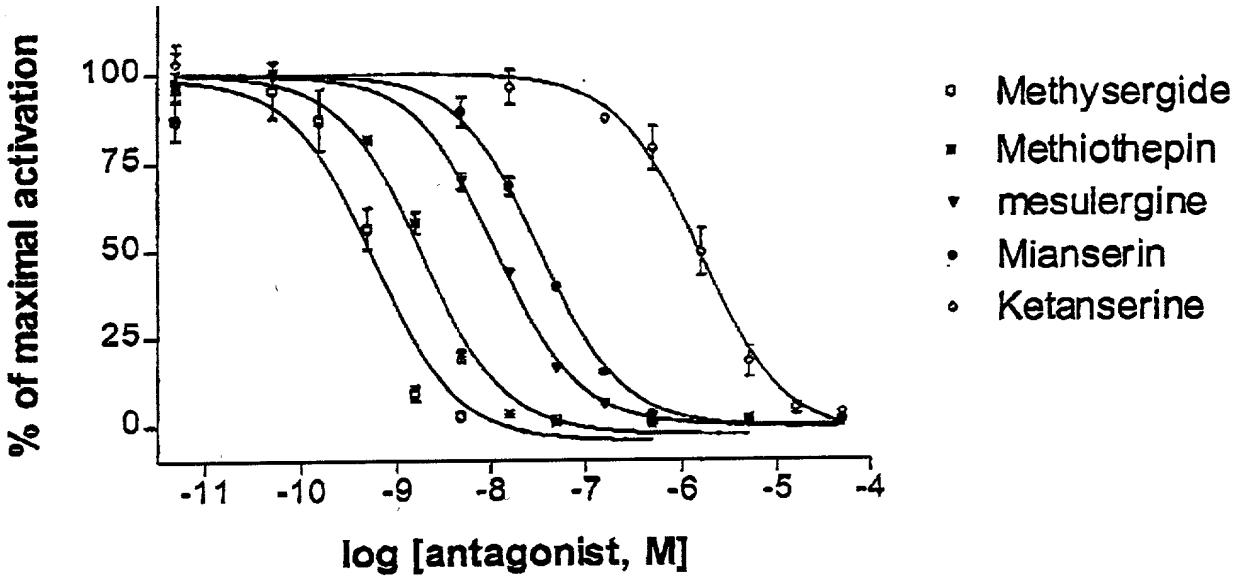
% of maximal activation
100
75
50
25
0
-11
-10
-9
-8
-7
-6
-5
-4
log [antagonist, M]
Methysergide
Methiothepin
mesulergine
Mianserin
Ketanserine

FIG. 6

Declaration and Power of Attorney for Patent Application

Déclaration et Pouvoirs pour demandes de brevet

French Language Declaration

En tant que l'inventeur nommé ci-après, je déclare par le présent acte que :

Mon domicile, mon adresse postale et ma nationalité figurant ci-dessous à côté de mon nom,

Je crois être le premier inventeur original et unique (si un seul nom est mentionné ci-dessous), ou l'un des premiers co-inventeurs originaux (si plusieurs noms sont mentionnés ci-dessous) du sujet revendiqué, pour lequel une demande de brevet a été déposée concernant l'invention intitulée :

et dont les caractéristiques sont fournies ci-joint à moins que la case suivante n'ait été cochée :

○ a été déposé le
sous le numéro de Demande des Etats-Unis ou
sous le numéro de demande internationale
PCT et modifiée le
(le cas échéant).

Je déclare par le présent acte avoir passé en revue et pris connaissance du contenu des caractéristiques ci-dessus, revendications comprises, telles que modifiées par tout amendement dont il aura été fait référence ci-dessus.

Je reconnais de voir divulguer toute information pertinente à l'examen de cette demande, comme le définit le Titre 37, §1.56 du Code fédéral des réglementations.

As a below named inventor, I hereby declare that :

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled :

HIGH-THROUGHPUT SCREENING DIAGNOSTIC AND/OR DOSAGE METHOD OF AN AGONIST AND/OR AN ANTAGONIST FOR A CALCIUM-COUPLED RECEPTOR.

the specification of which is attached hereto unless the following box is checked :

● was filed on July 6, 1999
as ~~United States Application Number~~ or PCT International Application Number
PCT/BE99/00087 and was amended on
(if applicable).

U.S. National Phase filed Jan. 8, 2001
assigned U.S. Serial No. 09/743,405

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentibility as defined in Title 37, Code of Federal Regulations, § 1.56.

French Language Declaration

Je revendique par le présent acte avoir la priorité étrangère, en vertu du Titre 35, § 119 du Code des Etats-Unis, sur toute demande étrangère de brevet ou certificat d'inventeur figurant ci-dessous et ai aussi pris connaissance de toute demande étrangère de brevet ou de tout certificat d'inventeur ayant une date de dépôt précédant celle de la demande à propos de laquelle une priorité est revendiquée.

I hereby claim foreign priority under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior foreign applications
Demande(s) de brevet antérieure(s)

(Number) (Numéro)	(Country) (Pays)	Priority claimed Droit de priorité revendiqué ● Yes / Oui ○ No / Non
98870149.6	E.P.	
(Day/Month/Year Filed) (Jour/Mois/Année de dépôt)		
6 July 1998		● Yes / Oui ○ No / Non
(Number) (Numéro)	(Country) (Pays)	
		○ Yes / Oui ○ No / Non
(Number) (Numéro)	(Country) (Pays)	
		○ Yes / Oui ○ No / Non

Je revendique par le présent acte tout bénéfice, en vertu du Titre 35, § 120 du Code des Etats-Unis, de toute demande de brevet effectuée aux Etats-Unis figurant ci-dessous et, dans la mesure où le sujet de chacune des revendications de cette demande de brevet n'est pas divulgué dans la demande américaine préalable, en vertu des dispositions de premier paragraphe du Titre 35, § 112 du Code des Etats-Unis, je reconnais devoir divulguer toute information pertinente à la demande de brevet comme défini dans le Titre 37, § 1.56 du Code fédéral des réglementations, dont j'ai pu disposer entre la date de dépôt de la première demande et la date de dépôt de la demande nationale ou PCT internationale :

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentibility as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application :

(Application Serial No.) (No. de série de la demande)	(Filing date) (Date de dépôt)	(Statut) (Breveté, en attente, annulé)	(Status) (Patented, pending, abandoned)
(Application Serial No.) (No. de série de la demande)	(Filing date) (Date de dépôt)	(Statut) (Breveté, en attente, annulé)	(Status) (Patented, pending, abandoned)

Je déclare par le présent acte que toute déclaration ci-incluse est, à ma connaissance, véridique et que toute déclaration formulée à partir de renseignements ou de suppositions est tenue pour véridique; et de plus, que toutes ces déclarations ont été formulées en sachant que toute fausse déclaration volontaire ou son équivalent est passible d'une amende ou d'une incarcération, ou des deux, en vertu de la Section 1001 du Titre 18 du Code des Etats-Unis et que de telles déclarations volontairement fausses risquent de compromettre la validité de la demande de brevet ou du brevet délivré à partir de celle-ci.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful and false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.

French Language Declaration

POUVOIRS : En tant que l'inventeur cité, je désigne par la présente l'(les) avocat(s) et/ou agent(s) suivant(s) pour qu'il(s) poursuive(nt) la procédure de cette demande de brevet et traite(nt) toute affaire avec le Bureau des brevets et marques s'y rapportant.
(mentionner le nom et le numéro d'enregistrement)

POWER OF ATTORNEY : As named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and trademark Office connected there with.

(list name and registration number)
The registrants of KNOBBE, MARTENS, OLSON & BEAR LLP, 620 Newport Center Drive, 16th Fl., Newport Beach, CA 92660

Adresser toute correspondance à :

Send Correspondence to : Daniel E. Altman
Registration No. 34,115
620 Newport Center Drive, Ste. 16
Newport Beach, CA 92260

Adresser tout appel téléphonique à :
(nom et numéro de téléphone)

Direct Telephone Calls to :
(name and telephone number)

(949) 760-0404
Customer No. 20,995

Nom complet de l'unique ou premier inventeur	Full name of sole or first inventor
1-00	***DUPRIEZ Vincent***
Signature de l'inventeur Date	Inventor's signature Date
	Oct 12, 2000
Domicile	Residence ***Avenue de Roodebeek 229*** ***B-1030 BRUSSELS*** ***BELGIUM*** BEX
Nationalité ***Belgian***	Citizenship ***Belgian***
Adresse postale	Post Office Address ***Avenue de Roodebeek 229*** ***B-1030 BRUSSELS*** ***BELGIUM***

(Fournir les mêmes renseignements et la signature de tout co-inventeur supplémentaire)

(Supply similar information and signature for any subsequent joint inventor)

Nom complet du second co-inventeur, le cas échéant 2-00	Full name of second joint inventor, if any ***DETHEUX Michel***
Signature du second inventeur Date	Second inventor's signature Date Oct 12, 2000
Domicile	Residence ***Rue Charles de Loupoigne 13/102*** ***B-1348 LOUVAIN-LA-NEUVE*** ***BELGIUM*** BEX
Nationalité ***Belgian***	Citizenship ***Belgian***
Adresse postale	Post Office Address ***Rue Charles de Loupoigne 13/102*** ***B-1348 LOUVAIN-LA-NEUVE*** ***BELGIUM***

Nom complet du troisième co-inventeur, le cas échéant 3-00	Full name of third joint inventor, if any ***PARMENTIER Marc***
Signature du second inventeur Date	Third inventor's signature Date Oct 13, 2000
Domicile	Residence ***Chaussée d'Uccle 304*** ***B-1630 BRUSSELS*** ***BELGIUM*** BEX
Nationalité ***Belgian***	Citizenship ***Belgian***
Adresse postale	Post Office Address ***Chaussée d'Uccle 304*** ***B-1630 BRUSSELS*** ***BELGIUM***

Nom complet du troisième co-inventeur, le cas échéant	Full name of third joint inventor, if any
Signature du second inventeur Date	Third inventor's signature Date
Domicile	Residence
Nationalité	Citizenship
Adresse postale	Post Office Address